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Complex Alternative RNA Processing Generates an Unexpected Diversity of Poly(A) Polymerase Isoforms

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Multiple forms of poly(A) polymerase (PAPs I, II, and III) cDNA have previously been isolated from bovine, human, and/or frog cDNA libraries. PAPs I and II are long forms of the enzyme that contain four functional domains: an apparent ribonucleoprotein-type RNA-binding domain, a catalytic region that may be related to the polymerase module, two nuclear localization signals (NLSs 1 and 2), and a C-terminal Ser/Thr-rich region. PAP III would encode a truncated protein that lacks the NLSs and the S/T-rich region. To investigate further the structure and expression of these forms, we isolated the mouse PAP gene and an intronless pseudogene from a mouse liver genomic library. The structure of the gene indicates that different forms of PAP are produced by alternative splicing (PAPs I and II) or by competition between polyadenylation and splicing (PAP III). The pseudogene appears to reflect yet another form of long PAP, which we call PAP IV. Mouse PAP III and two additional truncated forms, PAPs V and VI, which would be produced by use of poly(A) sites in adjacent introns, were also isolated from a mouse brain cDNA library. RNase protection and reverse transcription-PCR analyses showed that PAP II, V, and VI are expressed in all tissues tested but that PAP I and/or IV and III are tissue specific. However, immunoblot analysis detected only the long forms, raising the possibility that the short-form RNAs are not translated. Purified recombinant baculovirus-expressed PAPs were tested in several in vitro assays, and the short forms were found to be inactive. We discuss the possible significance of this complex expression pattern.

An enzyme activity able to polymerize AMP residues onto an RNA primer was first described over 30 years ago (9). Subsequent studies have described the purification and characterization of poly(A) polymerases (PAPs) and other factors involved in polyadenylation from many different sources. It is now well established that nuclear polyadenylation occurs in a two-step reaction (reviewed in references 19 and 21): a pre-mRNA is first endonucleolytically cleaved at the site of polyadenylation, which is usually located 10 to 30 nucleotides (nt) downstream of the polyadenylation signal, AAUAAA, and a poly(A) stretch of 200 to 250 nt is subsequently synthesized onto the 3′ end of the cleavage product.

Multiple factors participate in the 3′ end processing reaction. Cleavage-polyadenylation specificity factor (CPSF), which consists of subunits with sizes of 160, 100, and 73 kDa and possibly a 30-kDa polypeptide, recognizes AAUAAA (3, 27). CPSF is required for both cleavage and poly(A) addition. The 160-kDa subunit is probably responsible, at least in part, for contacting AAUAAA (28). Cleavage stimulation factor (CstF) was first identified as an activity required for efficient cleavage but not for poly(A) addition. CstF is composed of three subunits with sizes of 77, 64, and 50 kDa (39). The 64-kDa subunit of CstF contains an RNA-binding domain (RBD) in the N terminus and binds preferentially to GU-rich sequences downstream of AAUAAA (20, 36, 38). CPSF, or specifically its 160-kDa subunit, enhances this interaction (27, 28). The 77-kDa subunit bridges, via protein-protein interactions, the other two CstF subunits and also binds the 160-kDa subunit of CPSF (28, 37). Sequence analysis indicates that 77K is the human homolog of the *Drosophila* suppressor of forked [su(f)] protein. Mutations in *su(f)* can enhance or suppress the effects of transposable element insertion, very likely because of changes in polyadenylation (26, 37). Cleavage factors I and II are likely to form the endonuclease responsible for the cleavage of pre-mRNA (41). PAP is nonspecific by itself and is capable of synthesizing poly(A) tracts onto virtually any RNA primers in the presence of Mn^{2+}. However, PAP becomes specific for AAUAAA in the presence of Mg^{2+} and CPSF (29, 44, 45). The 160-kDa subunit of CPSF also interacts with PAP, perhaps providing a mechanism for linking CPSF and PAP (28). It is noteworthy that PAP is also required for efficient cleavage of many pre-mRNAs in vitro (for reviews, see references 19 and 21).

Cloning and expression of PAP cDNAs have begun to provide a great deal of information regarding the function and possible regulation of PAP (2, 14, 29, 42, 45). The initial characterization of bovine PAP cDNAs (termed PAP I and PAP II) (29) revealed several intriguing features, such as the presence of sequences at the amino terminus suggesting a ribonucleoprotein-type RBD followed by motifs resembling the so-called polymerase module (PM) found in template-dependent polymerases. Toward the carboxyl terminus are two nuclear localization signals (NLSs 1 and 2) followed by a nearly 200-residue region highly enriched in serine and threonine residues. Functional analyses have shown that the RBD and the PM-like domains are essential but not sufficient for catalytic activity. An ~270-residue stretch of sequence C terminal to the PM region is also required for specific and nonspecific polyadenylation (30). NLSs 1 and 2 are required for the localization of PAPs I and II into the nucleus. In addition, NLS 1 appears to be involved in the CPSF-PAP interaction, as mutations in this region disrupted specific but not nonspecific polyadenylation (30). PAP II may be the major form of the enzyme, as it was isolated from bovine, human, and frog cDNA libraries (2, 29, 45), while PAP I has only been isolated from a bovine cDNA library (29). PAP III, a third form of PAP mRNA, was isolated from human (30, 45) and *Xenopus* cDNA libraries (2, 14). PAP III would encode a truncated PAP lacking the NLS and S/T-rich region and, on the basis of mutational analyses (30), would

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not be expected to be active in specific and nonspecific polyadenylation assays in vitro. Consistent with this expectation, human PAP III has not been reported to be active (45), although a recombinant version of the frog enzyme was suggested to have low activity in a nonspecific assay (14).

Regulation of the length of poly(A) on specific maternal mRNAs appears to be a common means of translational activation in oocytes and early embryos (for a review, see reference 47). Cytoplasmic addition of poly(A) accompanies activation of many maternal mRNAs, whereas removal of poly(A) accompanies the inactivation of others (23, 32, 34, 35). The issue of whether cytoplasmic and nuclear polyadenylations are catalyzed by similar factors has been raised. Fox et al. (12) have shown that the cytoplasmic polyadenylation reactions that occur during frog oocyte maturation can be reconstituted in vitro by two fractions derived from a frog egg extract: one fraction contains an RNA binding activity and the other contains a PAP activity. The PAP fractions can be replaced by recombinant or purified bovine PAP, presumably nuclear in origin. In addition, a PAP cDNA isolated from a *Xenopus* ovary cDNA library, which corresponds to the maternal mRNA in a frog oocyte, is similar to PAP II isolated from bovine and human cDNA libraries (2).

In this report, we describe the isolation and structure of the mouse PAP gene and an intronless pseudogene. The structure of the genes indicates that the multiple forms of PAP mRNA can be produced by alternative splicing. Two more truncated forms of PAP were also isolated from a mouse brain cDNA library. Reverse transcription-PCR (RT-PCR), RNase protection, and immunoblotting assays were carried out to determine the expression of PAPs in different tissues, and evidence for complex expression patterns was obtained. Recombinant baculovirus-expressed PAPs were also tested for polyadenylation activity in vitro.

MATERIALS AND METHODS

Library screening. The mouse genomic library was in the Lambda FIX II vector (Stratagene). A total of 2.4×10^4 plaques were plated on a 150-mm-diameter petri dish, and 21 plates were prepared. The screening procedure was carried out by following the manufacturer's instructions. Bovine PAP I cDNA was used as a probe and was labeled by random priming (10). The 5′ and 3′ probes were prepared from bovine PAP II (29). The 5′ probe was a 352-bp fragment digested with *Pst*I (bp 172 to 523). The 3′ probe was a 277-bp fragment digested with *Eco*NI and *Eco*RI (bp 1915 to 2192). The prehybridizations and hybridizations were done as described by Sambrook et al. (33). The filters were incubated at 55°C with the probe at 2×10^6 counts per ml in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's reagent–0.5% sodium dodecyl sulfate (SDS)–100 μg of single-stranded salmon sperm DNA per ml (Sigma). Thirty positive clones were isolated from the first round of screening, and the 14 strongest clones were subjected to the second and third rounds of screening. Three clones contained the intronless mouse pseudogene, and the other 11 clones were overlapping fragments of the functional PAP gene. The gene fragments were subcloned into pBluescript vectors. The exons were identified by Southern blot analyses, and the subclones that contained exons were sequenced by the Sanger method.

The mouse brain cDNA library was in the Lambda ZAP II vector (Stratagene). The screening procedures were the same as those described above, and the mouse pseudogene was used as a probe. Ten positive clones were purified. The pBluescript plasmids were excised in vivo as described by the manufacturer, and the 10 clones were sequenced. Four clones, 7A, 11C, 1B, and 3A, were PAP III. 1B and 3A were identical clones starting from nt 435 in the open reading frame (taking the A of start codon ATG as nt 1). 7A and 11C were independent clones starting from nt −171 and −88 in the 5′ untranslated region (UTR), respectively. Two clones, 13C and 7B, were PAP V, starting from nt 42 and 582, respectively. An intact PAP V cDNA was constructed by fusing the 3′ portion of clone 13C to the 5′ portion of clone 11C. The other four clones, 1A, 11B, 11A, and 5A, were PAP VI. 11A and 5A were identical clones staring from nt −22. 1A and 11B were independent clones starting from nt −149 and −146, respectively. All the clones carried 16- to 19-nt poly(A) tails downstream of the polyadenylation signal, AATAAA.

RNase protection assays. Mouse tissues were homogenized, and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method (6). HeLa cell cytoplasmic RNA was isolated as described by Fu and Manley (13). The RNase protection assays were performed by basically following the procedures described by Ausubel et al. (1). Total RNA (20 μg) was used in each assay, and the hybridizations were done at 50°C overnight. Antisense probes (1 and 2) were transcribed from the cDNA of mouse or human PAP III with T7 RNA polymerase (Promega). [α-^{32}P]CTP (800 Ci/mmol; ICN) was used to label the probes. Probe 1 was complementary to nt 667 to 1216 in PAP III (nt 1116 to 1216 were encoded by intron 12) and was purified from a 5% polyacrylamide–urea gel. Probe 2 was complementary to nt 667 to 931 and was purified by two sequential ethanol precipitations.

RT-PCR. Total or cytoplasmic RNA (2 μg) was primed with 0.5 μg of random hexamers (Boehringer Mannheim) and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega). One-eighth of the reverse transcription product was used for PCR. For primers A and B, the annealing temperature was 50°C for mouse tissues and 55°C for HeLa cells. For primers A and C, the annealing temperature was 55°C for all tissues. A total of 35 cycles of 94°C for 45 s, 50 or 55°C for 45 s, and 72°C for 45 s were carried out. In the last cycle, the products were kept at 72°C for an additional 5 min. The sequence of primer A was 5′ GCATTCCTCAAACTGCCACAC 3′ (nt 1781 to 1800), that of primer B was 5′ GCAGGGAGAGCAGGGATATC 3′ (complementary to nt 2151 to 2171), and that of primer C was 5′ CTGAGAGGCCAACAGAG-AAG 3′ (complementary to nt 2042 to 2061). All primers were designed at the region where mouse and bovine PAP cDNAs are 100% identical at the nucleotide level.

Southern blot analysis. The products of RT-PCR were loaded onto a 2.7% agarose gel, transferred to nitrocellulose, and subjected to hybridization as described previously (33). Probe 1 was prepared from a subclone of the mouse PAP gene. The construct was digested with *Hin*dIII and *Pst*I, and the 800-bp fragment containing exon 20 was eluted from an agarose gel and purified by phenol extraction and ethanol precipitation (33). Probe 2 was prepared from the cDNA of bovine PAP I (29). The construct was digested with *Spe*I and *Sac*I, and the 490-bp fragment containing exons 18, 19, and 21 (nt 1616 to 2106) was purified as described above. The probes were labeled by random priming.

Baculovirus-expressed PAPs. The cDNAs of mouse PAPs III, V, and VI were initially subcloned into pET-14b vectors (Novagen) between the *Nde*I and *Bam*HI sites. The cDNAs were then excised from the pET-14b vectors and subcloned into the pEVmXIV vectors between the *Bgl*II and *Eco*RI sites. All three constructs contain six consecutive histidines (derived from the pET-14b vector) in a 10-residue segment upstream of the start codon. Recombinant baculoviruses encoding PAPs III, V, and VI were purified, and the titers of the virus were determined by standard procedures (22). Sf 21 cells were infected with 1 PFU of recombinant baculovirus per cell, and recombinant PAP proteins were purified essentially as described by Foukal et al. (11). Briefly, following a 42-h incubation at 27°C, the cells were lysed in 50 mM Tris (pH 8.0)–150 mM NaCl–1% Nonidet P-40–0.1% aprotinin–0.35 mM phenylmethylsulfonyl fluoride–40 mM imidazole. Lysates were collected and incubated on ice for 15 min, and then they were centrifuged at 12,000 × *g* for 10 min. The supernatants were collected and rocked for 1 h with Ni^{2+}-agarose (Qiagen). Beads were washed four times with RIPA buffer (150 mM NaCl, 50 mM Tris [pH 7.2], 1.0% Nonidet P-40, 2% sodium deoxycholate, 0.1% SDS) and eluted batchwise in an equal volume of 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5)–0.35 mM phenylmethylsulfonyl fluoride–0.1% aprotinin–200 mM imidazole–20% glycerol. The eluted proteins were dialyzed against buffer D [20 mM Tris (pH 8.4), 50 mM $(NH_4)_2SO_4$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.04% aprotinin, and 20% glycerol], aliquoted, and stored at −70°C.

Immunoaffinity purification of polyclonal antibodies. The ligand used in immunoaffinity purification was bacterially expressed bovine PAP I. PAP I cDNA was subcloned into pET-14b vector, and *Escherichia coli* BL21 was used for transformation. After growing to an optical density at 595 nm of 0.3 at 37°C, the cells were induced with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 30°C for 0.5 h. Rifampin (U.S. Biochemicals) was subsequently added to a final concentration of 150 μg/ml, and the cells were grown at 30°C for an additional 2.5 h. Histidine-tagged PAP I was purified with a Ni^{2+}-agarose column under denaturing conditions as described by the manufacturer (Qiagen). Purified PAP I was coupled to CNBr-activated Sepharose 4B (Pharmacia) by following the manufacturer's instructions. The beads with the bound antigen were transferred to a column and washed with 10 bed volumes of 10 mM Tris (pH 7.5), 100 mM glycine (pH 2.5), 10 mM Tris (pH 8.8), 100 mM triethylamine (pH 11.5; prepared fresh), and 10 mM Tris (pH 7.5). Polyclonal rabbit sera raised against purified recombinant PAP I (11) were diluted 10 times with 10 mM Tris (pH 7.5) and passed through the column 4 times. The washing of the column, elution, and dialysis of antibodies were carried out exactly as described by Harlow and Lane (18).

Western blot (immunoblot) analysis. HeLa cell whole-cell extracts were prepared as described by Sambrook et al. (33), except that no bromophenol blue was added in the 2× SDS gel loading buffer. The protein concentrations were measured by the Bradford method (Bio-Rad). The filters were incubated with the primary antibodies (immunoaffinity purified [see above]) at a dilution of 1:50 for 2 h and, after an intensive washing, were incubated with the secondary antibody (horseradish peroxidase [Cappel]) at a dilution of 1:5,000 for another 2 h. The signals were detected with the enhanced chemiluminescence Western blotting detection reagents (Amersham) by following the manufacturer's instructions.

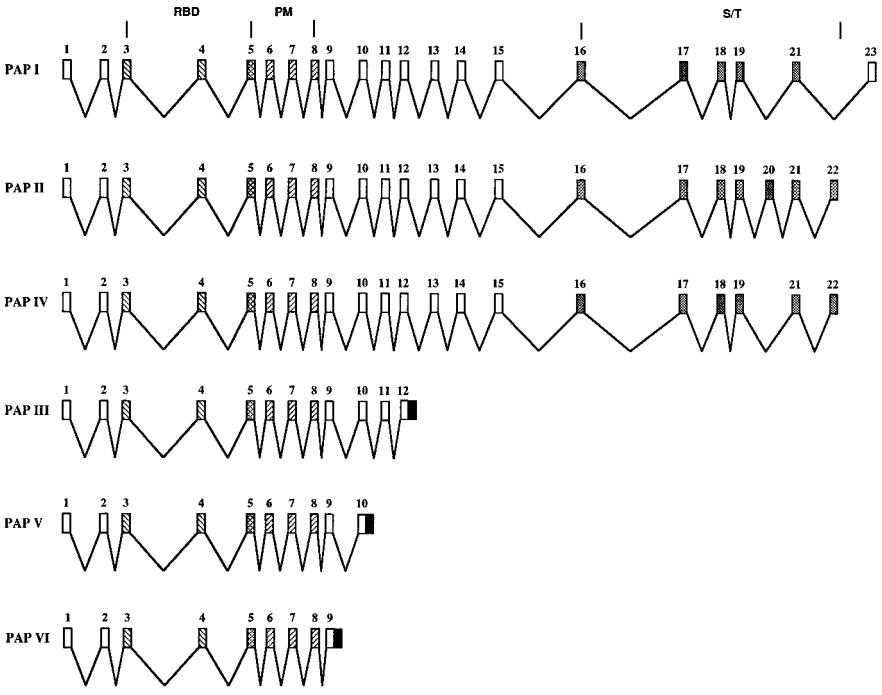

FIG. 1. Structures of the alternatively spliced forms of PAP. The numbered open boxes stand for exons, and splicing is indicated by angled lines between exons. The locations of the RBD, PM, and S/T-rich region (S/T) are indicated at the top of the figure. The solid boxes in PAPs III, V, and VI indicate the residues encoded by introns 12, 10, and 9, respectively.

In vitro polyadenylation assays. Plasmid pG3SVL-A, which contains the simian virus 40 late polyadenylation site, was digested with *Xho*I and used as a template to synthesize ^{32}P-labeled RNA substrate as described previously (28). In vitro nonspecific polyadenylation assays were carried out in a final volume of 25 μl under the following conditions: 0.5 mM $MnCl_2$, 2.5% polyvinyl alcohol, 1 mM ATP, 10 mM Tris (pH 8.4), 25 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, and 0.25 mM dithiothreitol. Totals of 1.6 pmol of each PAP and 0.5 pmol of substrate were added to each reaction. After an incubation at 30°C for 30 min, the reaction products were extracted, precipitated, and loaded onto a 5% polyacrylamide–urea gel. When oligo$(A)_5$ was used as a substrate (29), the reaction was carried out in a final volume of 25 μl under the following conditions: 0.5 mM $MnCl_2$, 0.3% polyvinyl alcohol, 0.1% bovine serum albumin (BSA), 0.1 mM ATP, 1 μCi of [α-^{32}P]ATP, 10 mM Tris (pH 8.4), 25 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, and 0.25 mM dithiothreitol. Totals of 1.6 pmol of each PAP and 2.0 pmol of oligo$(A)_5$ were added to each reaction. After an incubation at 30°C for 60 min, the reaction products were spotted onto DE-81 paper (Whatman), dried at room temperature, washed with Na_2HPO_4 (pH 7.0) for 5 min (three times), washed briefly with 70% ethanol, and dried at room temperature. The incorporation of [α-^{32}P]ATP was measured by scintillation counting.

RESULTS

The mouse PAP gene and a pseudogene. Three forms of PAP cDNA were previously isolated from bovine or human cDNA libraries: PAPs I, II, and III (29, 45). It was speculated that these forms might be the products of alternative splicing (29). To test this possibility and to learn more about the intron-exon structure of PAP, a mouse genomic library was screened, with the cDNA of bovine PAP I being used as a probe. Fourteen positive clones (see Materials and Methods) were analyzed by restriction mapping and Southern blot analyses, and 11 clones were overlapping and covered 45 kb. The subclones that contained exons were identified by Southern blot analyses and sequenced. Twenty-one exons from exon 2 to 22 were identified in the 45-kb region (Fig. 1). Exon 1, which was beyond the region that we subcloned, would encode the 5′ UTR of PAP and the first three residues of the open reading frame. The RBD is located in exons 3, 4, and 5; the PM is located in exons 5, 6, 7, and 8; NLS 1 is located in exon 16; and NLS 2 is located in exon 19. The S/T-rich region is located from exon 16 to 22 (Fig. 1). PAP is very conserved, especially at the N-terminal part. Homology for mouse and bovine PAPs is 100% at the amino acid level from exon 3 to 12. NLS 2 is also conserved between bovine and mouse PAPs. In NLS 1, the second lysine in the NLS 1B motif (KKKK [30]) is changed to arginine.

As expected, PAPs I and II, which share the first 19 exons, are produced by alternative splicing (Fig. 1). In PAP II, exon 19 is followed by exons 20, 21, and 22. In PAP I, however, exon 20 is skipped. Exon 19 is spliced to exon 21, which is followed by a putative exon 23. We were unable to identify exon 23, because an appropriate probe was not available. In bovine PAP I, putative exon 23 would encode the last two residues of the open reading frame and a long 3′ UTR.

Two additional probes were used to analyze the other three positive clones. The 5′ probe was a 352-bp fragment prepared from the cDNA of bovine PAP II (nt 172 to 523) (29). The 3′ probe was a 277-bp fragment also prepared from PAP II (nt 1915 to 2192). Surprisingly, the region that hybridized to both probes in the three positive clones could be narrowed down to 4 kb. The 4-kb fragment was subcloned and sequenced. This gene, which is intronless, is 84% identical to the functional mouse PAP cDNA (postulated from the mouse PAP gene) over 1,970 nt (from exon 2 to 19). Homology in the 5′ UTRs is somewhat lower, with 65% identity over 169 nt (see below). A single nucleotide deletion in the intronless gene corresponding to nt 306 in the functional PAP cDNA (taking the A of start codon ATG as nt 1) and resulting in a frameshift and premature termination was observed. Together, these observations suggest that the intronless gene is a processed pseudogene (43). The Ser/Thr-rich region diverges somewhat from that of the functional PAP cDNA, and multiple in- and out-of-frame nucleotide deletions were found. Although the homology in

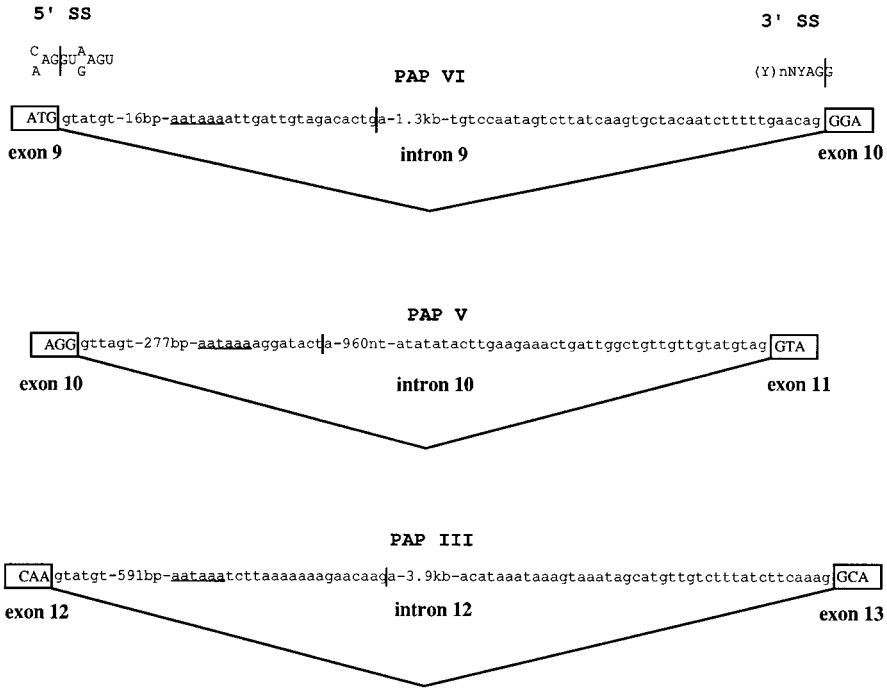

FIG. 2. PAPs III, V, and VI are generated by competition between polyadenylation and splicing. Exons are represented by open boxes, and sequences at the exon-intron junctions are shown. Consensus splice site sequences (SS) are shown at the top of the figure. The AATAAA hexanucleotides are underlined, and the locations of cleavage and polyadenylation sites are indicated by vertical rules. Usage of polyadenylation signals within introns 9, 10, and 12 will produce PAPs VI, V, and III, respectively, whereas usage of the adjacent splice sites (indicated by angled lines) will generate longer forms of PAP.

the 3′-terminal part is relatively low, a careful comparison of the pseudogene and the functional gene suggested that the pseudogene might represent another alternatively spliced form of PAP in which exon 20 is skipped and exon 19 is followed by exons 21 and 22 (Fig. 1). There is 75% identity over 220 nt in exon 19, 77% identity over 61 nt in exon 21, and 68% identity over 93 nt in exon 22. Processed pseudogenes (also referred to as processed retropseudogenes) are believed to arise by retroposition, in which an mRNA species is reverse transcribed into DNA copies and randomly inserted into the genome (5, 43, 46). It is likely that this pseudogene arose from another form of PAP mRNA, which we named PAP IV (Fig. 1 and see below).

Isolation of three truncated forms of PAP cDNA from a mouse brain cDNA library. We next screened a mouse brain cDNA library by using the pseudogene as a probe in the hope of isolating mouse PAP I, II, or IV cDNAs. Unexpectedly, all 10 clones that were isolated encoded truncated forms of PAP. Four represent the mouse counterpart of human PAP III, which terminates after exon 12. The other six clones were still shorter, two stopping after exon 10 (PAP V) and four stopping after exon 9 (PAP VI) (Fig. 1). The 5′ UTR is also present and conserved between mouse and bovine PAPs, with the regions being 79% identical over 153 nt and very GC rich.

An interesting common feature of the short forms is that they all appear to be produced by competition between polyadenylation and splicing (Fig. 2). All three forms utilize polyadenylation signals within intron 12, 10, or 9 (III, V, or VI, respectively). Usage of the polyadenylation signals will produce the short PAPs, while usage of the surrounding splice sites will result in longer forms of PAP. PAPs III, V, and VI each contain 3, 14, and 14 amino acids encoded by introns 12, 10, and 9, respectively, at their C termini (Fig. 1). All three introns contain an AAUAAA hexanucleotide. MacDonald et al. (20) have shown that the 64K subunit of CstF can be cross-linked to U-rich sequences 15 to 30 nt downstream of the cleavage sites in simian virus 40 late and adenovirus L3 pre-mRNAs. SELEX experiments have shown that the 64K RBD preferably binds to $G(U)_nG$ or $(GU)_n$ sequences ($n = 2$ to 4) (38). In PAP VI, the sequence 2 to 62 nt downstream of the cleavage site is very GT rich, and multiple sequences resembling $G(T)_nG$ were found. In PAP V, the GT-rich region is located 15 to 80 nt downstream of the cleavage site. Consecutive Ts and sequences resembling $G(T)_nG$ were also found. In PAP III, a TTATTT sequence beginning 21 nt downstream of the cleavage site was found, although no obvious GT-rich region within 60 nt was found. This result suggests that the PAP III polyadenylation signal is weak and may explain the relatively low level of PAP III mRNA detected in vivo (see below). The consensus splice site sequences are also indicated in Fig. 2. The intron 9, 10, and 12 sequences all contain reasonable matches, although the pyrimidine contents of the 3′ splice sites are somewhat low.

PAP I, II, and IV mRNAs are expressed in vivo in a tissue-specific manner. From the structure of the mouse PAP gene, we have shown that PAP I, II, and IV mRNAs can be generated by alternative splicing. To test if these forms are in fact produced in vivo, RT-PCR experiments were performed. Two primers, A and B (Fig. 3A; see Materials and Methods), were designed to discriminate between PAPs II and IV. Since primer B is complementary to 20 nt in exon 22, primers A and B will not amplify PAP I. Theoretically, 390- and 326-bp fragments will be amplified from PAPs II and IV, respectively. The cDNA of bovine PAP II was also amplified with primers A and B to produce a DNA size marker (lane 1 in the left panel of Fig. 3B). Total cell RNA isolated from mouse brain, intestine,

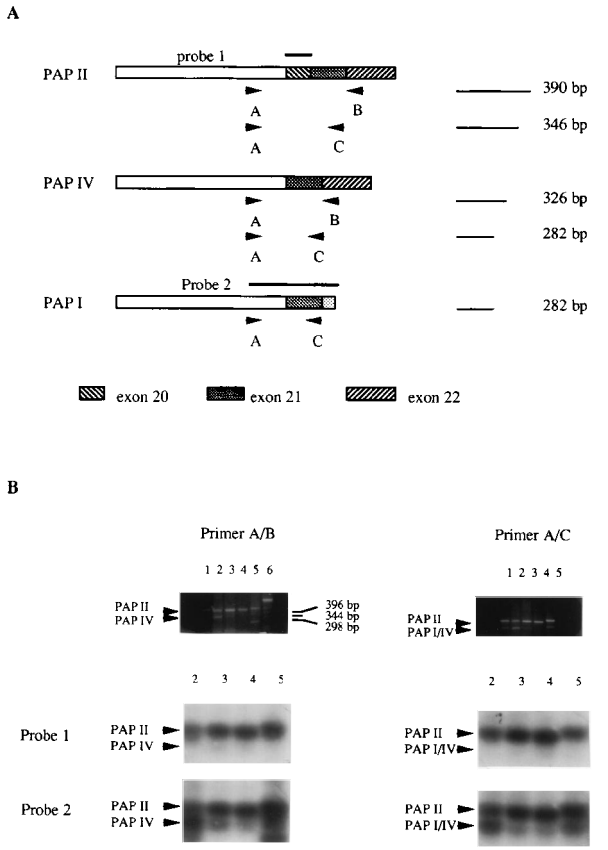

FIG. 3. Detection of PAP II, PAP IV, and/or PAP I mRNAs by RT-PCR. (A) A diagram of the RT-PCR experiments. Primers A and B amplify fragments with sizes of 390 and 326 bp for PAPs II and IV, respectively. Primers A and C amplify fragments with sizes of 346 bp for PAP II and 282 bp for PAP IV and/or PAP I, respectively. Probe 1 was prepared from exon 20 (see text) and hybridizes to PAP II only. Probe 2 contains exons 19 and 21 and hybridizes to PAPs I, II, and IV. (B) Southern blot analyses of RT-PCR products. Lanes 1, DNA size markers amplified from the cDNA of PAP II (Primer A/B) or from the cDNAs of PAP II and I (Primer A/C). Total RNAs isolated from mouse brain (lanes 2), intestine (lanes 3), and liver (lanes 4) and cytoplasmic RNA isolated from HeLa cells (lanes 5) were tested. A DNA ladder (GIBCO BRL) was also loaded as a size marker (Primer A/B, lane 6). The RT-PCR products were separated on a 2.7% agarose gel (upper panels), transferred to nitrocellulose, and hybridized to probe 1 (middle panels). The same filter was stripped of probe 1 and hybridized to probe 2 (lower panels). The expected positions of PAP II, IV, and/or I are indicated by arrowheads.

and liver and cytoplasmic RNA isolated from HeLa cells were analyzed. The amplification products were separated on a 2.7% agarose gel. In brain cells (Fig. 3B, lanes 2), two major bands were detected: one migrated as predicted for PAP II, and the other was close to PAP IV (326 bp). In intestine, liver, and HeLa cells (Fig. 3B, lanes 3, 4, and 5), PAP II appeared to be the major form. However, low levels of PAP IV were also detectable in intestine and HeLa cells if more PCR products were loaded (data not shown). Negative controls in which the RNA samples from different tissues were subjected to PCR without reverse transcription were performed, and no bands were detected when the same amounts of PCR products were analyzed (data not show). In HeLa cells (Fig. 3B, lanes 5), the two amplified fragments migrated somewhat more slowly than the mouse and bovine counterparts. Thuresson et al. (42) have reported that 18 nt (between nt 1883 and 2000) are tandemly duplicated in human but not bovine PAP mRNA. This 18-bp duplication would be included in the amplified fragments by primers A and B (also A and C [see below]). Our results are thus consistent with their observation.

To confirm that the PCR fragments detected were in fact derived from PAP II or PAP IV mRNA, Southern blot analyses were performed. The RT-PCR products were transferred to nitrocellulose and detected with two probes: probe 1 was derived from a subclone of the mouse PAP gene which contained exon 20, while probe 2 was prepared from the 3′-terminal part of bovine PAP I and contains exons 18, 19, and 21 (see Materials and Methods). Since exon 20 was skipped in PAP IV, probe 1 will not hybridize to the fragments amplified from it. However, probe 2 will hybridize to fragments amplified from both PAP II and PAP IV. The result is shown in the middle and lower panels of Fig. 3B. As expected, the 390-bp fragments (PAP II) hybridized to both probes, whereas the 326-bp fragments (PAP IV) hybridized to probe 2 only. A band with a size of ~260 bp was also observed in HeLa cells and showed slight hybridization to probe 2. This fragment was purified, subjected to a round of asymmetric amplification (17), and sequenced. The sequence of this fragment was 56% identical to that of bovine PAP over 190 nt (nt 1950 to 2120) (29), but it did not contain an open reading frame. Comparison with the sequences in the GenBank database did not indicate any significant homologies.

Primers A and B do not amplify PAP I because it does not contain exon 22. Primer C, which is complementary to 20 nt in exon 21, was designed to amplify PAP I. Primers A and C will amplify fragments with sizes of 342 bp for PAP II and 282 bp for PAPs I and IV (Fig. 3A). Although primer C will amplify both I and IV into fragments with the same sizes, we hoped that some insights into PAP I expression could be obtained by comparing the intensities of the 326-bp (PAP IV) and 282-bp (PAP IV plus PAP I) bands. Bovine PAP I and II cDNAs were amplified to produce DNA size markers (lane 1 in the right panel of Fig. 3B). The results obtained were similar to those obtained with primers A and B. In brain cells (Fig. 3B, lanes 2), two major fragments were amplified, and they migrated exactly as PAP II and PAPs I and IV. In intestine, liver, and HeLa cells (Fig. 3B, lanes 3, 4, and 5), PAP II was the major form. Low levels of PAPs I and IV were also detected in HeLa cells. The results were again confirmed by Southern blot analyses. As expected, the higher bands hybridized to both probe 1 and 2, while the lower bands hybridized to probe 2 only.

Together, these results suggest that PAP II is the major form in all tissues tested, although PAP IV is also predominant in brain tissues. We cannot tell whether PAP I was expressed at a significant level. Although the 282-bp bands (PAP IV plus PAP I) were slightly stronger than the 326-bp bands (PAP IV) in intestine and HeLa cells, the difference was not great enough to draw firm conclusions concerning PAP I expression. We cannot exclude the possibility that PAP I expression is limited to bovine PAP, although the structure of the mouse gene clearly provides the potential for its synthesis. However, our results do show that alternatively spliced forms of PAP mRNA are produced.

The truncated forms of PAP are also expressed at the mRNA level. To determine if the truncated forms of PAP are expressed, RNase protection assays were carried out. Two antisense probes, both derived from mouse or human PAP III cDNAs, were designed for this purpose. Probe 1 (Fig. 4A) covered 449 nt (spanning exons 8 to 12) in the open reading frame and 101 nt of the 3′ UTR of PAP III. After hybridization and RNase digestion, it will give protected fragments with sizes of 550 nt for PAP III mRNA and 449 nt for the long forms (I, II, and IV) of PAP mRNAs. The probe itself was ~596 nt in length and contained sequences from the pBluescript vector at

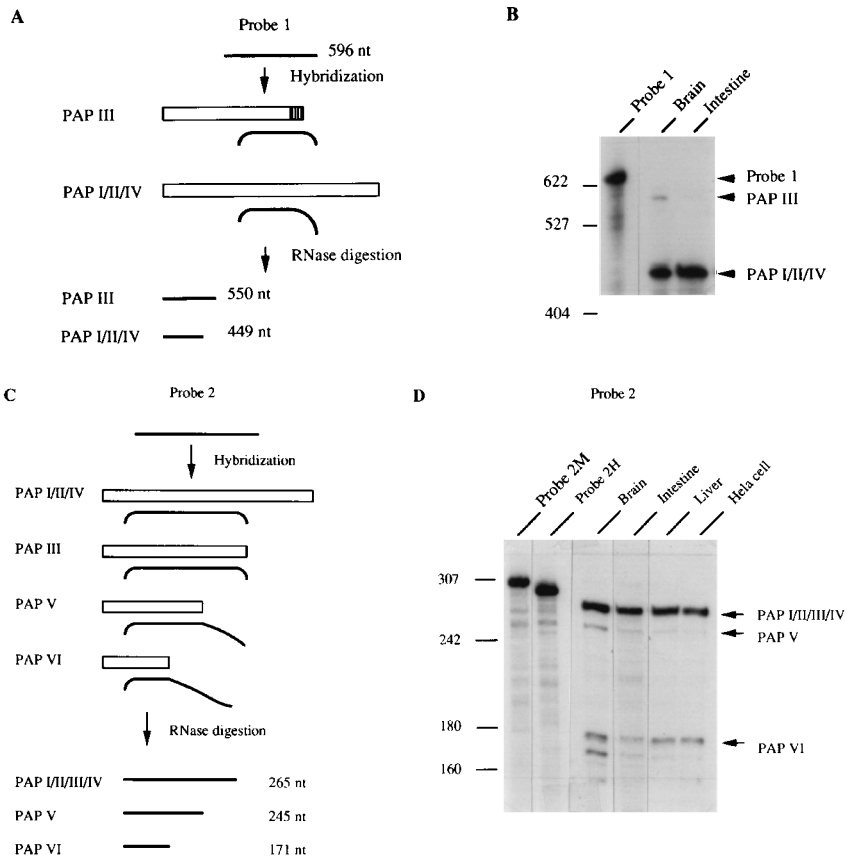

FIG. 4. Detection of PAP III, V, and VI and full-length PAP mRNAs by RNase protection. Panels A and C are diagrams of the RNase protection assays with probes 1 and 2. (A) Probe 1 was prepared from the cDNA of mouse PAP III (see text). The open boxes indicate PAP exons, and the striped box represents the sequences encoded by intron 12. The solid lines stand for labeled probes. (B) Total RNAs isolated from mouse brain and intestine were analyzed with probe 1. The RNase protection products were separated on a 5% polyacrylamide–urea gel. Labeled *Msp*I-digested pBR322 DNA was used as a DNA size marker (left). The positions of the probe, PAP III, and full-length PAP (I, II, and IV) are indicated by arrowheads on the right. (C) Diagram of the RNase protection assays performed with probe 2. Probe 2 was prepared from either mouse (probe 2M) or human (probe 2H) PAP III (see text). (D) Total RNAs isolated from mouse brain, intestine, and liver and cytoplasmic RNA isolated from HeLa cells were tested with probes 2M and 2H, respectively. DNA size markers (in nucleotides) are indicated on the left, and the positions of PAP I-II-III-IV, PAP V, and PAP VI are indicated by arrows on the right.

both ends. Total RNAs isolated from mouse brain and intestine were tested (Fig. 4B). PAP III mRNA was found to be expressed at relatively low levels compared with those of the long PAPs. Among the different tissues tested, brain tissue expressed the highest level of PAP III mRNA (liver and heart data are not shown). Probe 2 (spanning exons 8 to 11) (Fig. 4C) was designed to discriminate among the long forms of PAP (I, II, III, and IV; 265 nt) and PAPs V (245 nt) and VI (171 nt) mRNA. Total RNAs isolated from mouse brain, intestine, and liver and cytoplasmic RNA from HeLa cells were examined. In all four tissues, the long forms of PAP were again the most abundant, but PAP VI was also expressed at relatively high levels in the four tissues (Fig. 4D). Phosphor image scanning showed that the ratios (adjusted for length) of the 265-nt band to the 171-nt band in brain, intestine, liver, and HeLa cells were 2.1:1, 2.1:1, 2.0:1, and 2.3:1, respectively. PAP V mRNA was also detected in all four tissues at a level lower than that for PAP VI. Total and nuclear RNAs isolated from HeLa cells were also tested to determine the distribution of different forms in the nucleus and cytoplasm, but the results were similar to those obtained with the cytoplasmic RNAs (data not shown).

Since total mouse cell RNA was used in the RNase protection assays and the probes were derived from cDNAs, the protected fragments may also reflect partially spliced nuclear pre-mRNAs as well as or instead of mRNAs polyadenylated within the introns. However, we believe this is very unlikely. Northern (RNA) blot analyses of brain and liver poly(A)$^+$ RNA were also performed, and in each case, the species with the size predicted for the short forms of PAP mRNA was detected (data not shown). Also, RNase protection assays of mouse intestine and liver poly(A)$^+$ RNAs gave results identical to those obtained with total RNAs. An additional ~160-nt band was also observed in the four tissues, and the level was relatively higher in brain and intestine. However, this species likely reflects the product of degraded RNA or incomplete digestion of the probe, as it was not observed when the RNase protection assays were performed in poly(A)$^+$ RNA and occasionally appeared in the negative control (tRNA) (data not shown). Together, our results suggest that the truncated forms of PAP mRNA originally isolated as cDNAs from mouse brain and/or HeLa cells also exist in other tissues. Among the different forms of PAP mRNA, PAP II (in the case of brain, PAPs II and IV) is the major form and is followed by PAP VI and PAP V. PAP III mRNA is the least abundant form and the expression level is tissue dependent, with higher levels being found in brain than in the other tissues tested. No significant differences in the relative PAP VI and PAP V mRNA expres-

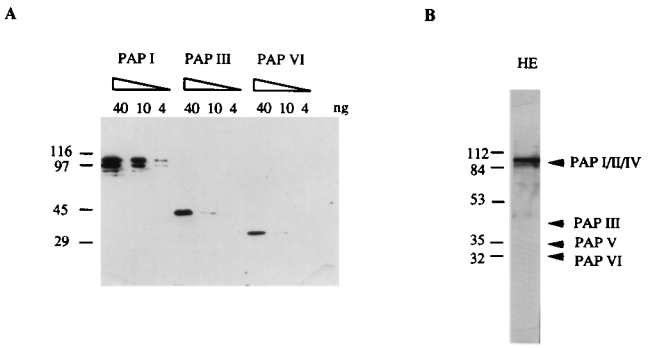

FIG. 5. Western blot analyses of recombinant PAPs and HeLa cell whole-cell extract. (A) Western blot analysis of baculovirus-expressed recombinant PAPs I, III, and VI separated on a 9% polyacrylamide–SDS gel. The protein concentrations were estimated by loading in parallel with different concentrations of BSA. For the convenience of visual comparison, the most hyperphosphorylated form (highest band) of PAP I was used for estimation, and thus the concentration of PAP I refers to this form. Protein size markers (in kilodaltons) are indicated on the left. (B) Western blot analysis of HeLa cell whole-cell lysate (35 μg) separated on a 10% polyacrylamide–SDS gel. Protein size markers (in kilodaltons) are indicated on the left, and the expected positions of the short forms of PAP are indicated by arrowheads on the right.

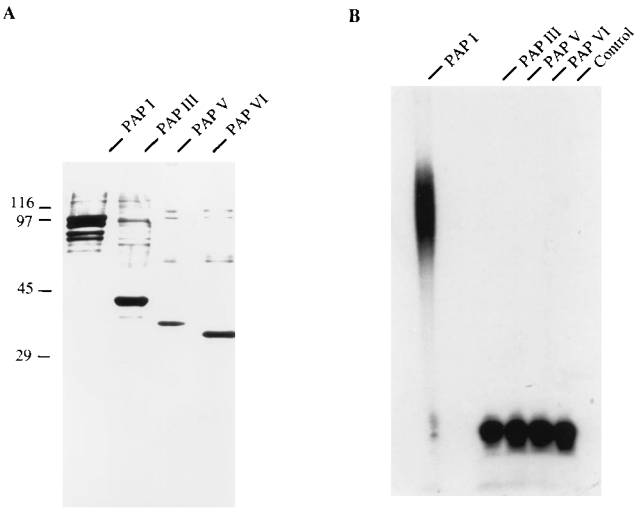

FIG. 6. The short forms of PAP are not active in nonspecific polyadenylation assays in vitro. (A) Baculovirus-expressed PAPs I, III, V, and VI were purified (see Materials and Methods) and separated on a 10% polyacrylamide–SDS gel. The proteins were visualized by silver staining. Protein size markers (in kilodaltons) are indicated on the left. (B) Nonspecific polyadenylation assays of recombinant PAPs I, III, V, and VI and control proteins (see the text). After extraction and precipitation, the reaction products were separated on a 5% polyacrylamide–urea gel and detected by autoradiography.

sion levels were detected among different tissues. It is noteworthy that the relative abundance of the short forms reflects the apparent strength of the polyadenylation signals.

Western blot analyses of HeLa cell whole-cell extract. The results presented above have shown that multiple forms of PAP mRNA exist in vivo. To test if all of these mRNAs are expressed as proteins, Western blot analyses were performed. Polyclonal antibodies raised against baculovirus-expressed PAP I (11) were affinity purified (see Materials and Methods) and used in the assays shown in Fig. 5. As a positive control, decreasing amounts of purified recombinant PAPs I, III, and VI were loaded onto an SDS-polyacrylamide gel and subsequently blotted onto nitrocellulose. The antibody recognized all forms tested, although the affinity for PAPs III and VI was about five times lower than that for PAP I. A possible explanation is that the N-terminal part of PAP is significantly more conserved than the C-terminal part and therefore likely less to be antigenic. Figure 5B is a Western blot analysis of HeLa cell whole-cell lysate separated on a 10% polyacrylamide–SDS gel. A band with a size of ~100 kDa which represents the long-form PAP(s) was detected. PAPs III, V, and VI would encode proteins with sizes of 41, 34, and 32 kDa, respectively. However, in this and other experiments, we have been unable to detect any of these short forms. These results suggest that although the short-form mRNAs are expressed in HeLa cells, the corresponding proteins may not be.

Recombinant short PAPs are not active. Previous mutagenic studies (29, 30) suggest that the short forms of PAP may be inactive in vitro. To address this issue directly, recombinant baculoviruses encoding PAPs I (11), III, V, and VI were constructed and the proteins were purified from infected insect cells. All forms were histidine tagged at their N termini and purified with Ni^{2+}-agarose columns (Fig. 6A; see Materials and Methods). First, nonspecific polyadenylation assays were performed. The primer used was an α-^{32}P-labeled, 30-nt RNA, and equal amounts of each PAP were added to the assays (see Materials and Methods). An uninfected insect cell lysate subjected to the same purification procedures as those for the infected cells was used as a negative control. RNAs were analyzed by gel electrophoresis (Fig. 6B), and no activity was observed with any of the short forms, although, as expected, substantial activity was detected with PAP I. Several additional RNA primers gave identical results (data not shown). Notably, given the possible role of the short forms of PAP in cytoplasmic polyadenylation, a substrate containing ~220 nt of the *Xenopus laevis* c-*mos* mRNA 3′ UTR (35) followed by 15 As was also tested with PAP III, and no activity was detected. Another nonspecific polyadenylation assay in which [α-^{32}P] ATP was the substrate and oligo$(A)_5$ was used as the primer (29) was also performed, and similar results were obtained, i.e., the short forms were completely inactive (data not shown). We also tested possible blocking (i.e., competitive inhibition) and nuclease activities of the short PAPs. In the blocking assays, increasing amounts of the short PAPs were added to reaction mixtures containing PAP I to determine if they can competitively inhibit the activity of the functional PAP. No effect on the polyadenylation efficiency or poly(A) length was observed when the short PAPs (PAPs III and VI) were added at molar concentrations of up to 10-fold higher than those of the functional PAP (data not shown). Nuclease activity assays in which the short PAPs were incubated under various conditions with α-^{32}P-labeled RNA primers containing poly(A) tails added by the functional PAP were also carried out. No nuclease activity was detected in the short PAPs, and the PAPs were not able to extend the poly(A) tails under conditions favorable to poly(A) synthesis (data not shown).

DISCUSSION

In this report, we described the structure of the mouse PAP gene and a related pseudogene. The PAP gene is unexpectedly complex, and a number of PAP mRNAs are produced by alternative splicing. One type of alternative splicing involves the inclusion or skipping of exon 20, the products of which are PAP II or PAPs I and IV, respectively. The existence of PAP IV was deduced from the structure of the intronless pseudogene. The difference between PAPs I and IV is whether the last exon, exon 22, is included during splicing. Recently, another long form of PAP cDNA in which exons 20 and 21 are skipped

and exon 19 is spliced to exon 22 was isolated from a human brain cDNA library (42a). The truncated PAPs (PAPs III, V, and VI) are the products of competition between polyadenylation and splicing: usage of polyadenylation signals within introns (introns 12, 10, and 9 for PAPs III, V, and VI, respectively) will produce the short forms of PAP, whereas usage of the downstream splicing sites will produce longer forms of PAP. RT-PCR and RNase protection assays showed that all the forms of PAP mRNA likely exist in vivo. PAPs II, V, and VI were detected in all tissues tested, whereas PAPs III, IV, and/or I are expressed in a tissue-dependent manner.

To our knowledge, PAP is the first polymerase reported to have alternative mRNA forms. Important questions are whether these forms are all translated into proteins and what their functions might be. The long PAPs (PAPs I, II, and IV) are active in in vitro assays (29, 30, 45). As initially suggested (29), the S/T-rich region has been shown to be a regulatory region in two aspects: phosphorylation and protein-protein interactions. Multiple potential protein kinase sites have been found in the S/T-rich region, including three consensus cyclin-dependent kinase (cdk) sites. PAPs I and II have been shown to be phosphorylated in vitro and in vivo by the Cdc2-cyclin B complex, which results in a dramatic reduction in both specific and nonspecific polyadenylation activity (11). Interestingly, the three cdk sites are all contained in exon 19 and are therefore present in PAPs I, II, and IV. However, other potential kinase sites also exist in the exons that are regulated by alternative splicing, which could determine whether PAP becomes a target of certain kinases. The U1 small nuclear ribonucleoprotein particle-specific A protein, U1A, has been reported to down regulate the polyadenylation of its own mRNA via binding to elements upstream of AAUAAA and interacting with PAP. The interaction region of PAP is in the C-terminal part of the S/T-rich region (4, 16). It is possible that specific interactions of PAP with regulatory proteins, as exemplified by U1A, are regulated by alternative splicing and/or phosphorylation.

The existence of the truncated forms of PAP is intriguing. The facts that these short forms lack the NLSs and that transfected or injected PAP III localizes to the cytoplasm (14, 30) suggest that the short forms could be involved in cytoplasmic polyadenylation. However, our observation that these forms are inactive in vitro argues against this, although we cannot exclude the possibility that additional factors allow the short PAPs to gain activity. A previous study reported the nonspecific activity of *X. laevis* PAP III expressed in *E. coli* as a glutathione *S*-transferase fusion protein, although the specific activity of glutathione *S*-transferase–PAP III was estimated to be 100-fold lower than that of the full-length PAP (14). It is conceivable that the activity detected in these assays was due to contamination with bacterial PAP, since the concentration of the fusion protein used was high. Although the frog PAP III was detected at the mRNA level in oocytes and ovaries (14), no evidence concerning its expression as a protein was reported. It is noteworthy that the mouse PAP VI and frog PAP III cDNAs do not contain 3′ UTRs. The stop codons are followed immediately by poly(A) tails, and the AAUAAA polyadenylation signals are therefore contained in the open reading frames (reference 14 and this paper).

Taking into consideration that the short-form mRNAs are produced by competition between polyadenylation and splicing, an intriguing possibility is that these RNAs reflect products of autoregulation by the functional PAPs. When functional PAPs are overexpressed, they may enhance the usage of the promoter-proximal polyadenylation signals by increasing cleavage efficiency. The observation that PAP is required for efficient cleavage of many pre-RNAs (7, 8, 15, 31, 40) is consistent with this model. A classic example of competition between polyadenylation and splicing is the switch from membrane-bound forms to secreted forms of immunoglobulin heavy chains during B-cell maturation (for a review, see reference 28a). It is possible that this switch is mediated by changes in the general mRNA processing machinery (28b).

It is noteworthy that PAP is not the only polyadenylation factor described to have truncated mRNA forms. Three forms of mRNA transcribed from the *su(f)* gene were detected in *Drosophila* embryos (26). One was a truncated form in which a polyadenylation signal in intron 4 (total, nine exons) was used. The short *su(f)* mRNA does not contain a stop codon: the reading frame continues to be open for translation to the position where it is polyadenylated, so the putative protein would have a polylysine track encoded by the poly(A) tail at its C terminus (26). It is likely that the truncated form of *su(f)* mRNA is nonfunctional, since a construct unable to synthesize this form is able to rescue *su(f)* mutants (48). Furthermore, the mRNA encoding the truncated form in several *su(f)* mutants is not evident or is less abundant than that in wild-type animals (26), suggesting that *su(f)* activity is required for its synthesis. The *Saccharomyces cerevisiae RNA14* gene, which bears homology to 77K and *su(f)* and is involved in cleavage and polyadenylation in yeast cells, was also reported to have truncated forms of mRNA (24, 25). However, no short forms of the human 77K mRNA have been detected (38).

The *Drosophila suppressor of white-apricot* [$su(w^a)$] gene is another example of autoregulation by RNA processing factors. The $su(s^a)$ gene encodes a protein containing an arginine/serine-rich region (RS domain), which is characteristic of splicing factors. Three forms of $su(w^a)$ transcripts were detected in vivo: 3.5, 4.4, and 5.2 kb. The protein coded for by the 3.5-kb transcript, which is the functional form, blocks splicing of the first or the first and second introns of its own pre-mRNA, and thus the nonfunctional 4.4- and 5.2-kb transcripts are formed (49). It may be typical of certain mRNA processing factors to autoregulate their own expression by negative feedback when they are overexpressed and thus nonfunctional or even when untranslated forms of mRNAs are produced. We are currently testing whether PAP autoregulates its synthesis in this manner.

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